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BIOLOGICAL BULLETIN

EFFECTS OF ACUTE ALCOHOLIZATION ON THE GERM CELLS OF FUNDULUS HETEROCLITUS.

WILSON GEE.

EMORY UNIVERSITY, OXFORD, GEORGIA.

CONTENTS.

INTRODUCTION.

This paper presents a study of the effects of short length treatments of the germ cells of *Fundulus heteroclitus* with various

concentrations of alcohol. The problem was at first undertaken with the idea of determining what might be the results of a direct treatment of the spermatozoa with alcohol. Early in the progress of the work, it became apparent that even in low concentrations the solutions used were markedly toxic to the unfertilized egg. There seems no practical way to separate the spermatozoa from the alcohol with which they are treated, and some of this material must come in contact with the eggs in the process of fertilizing them. So, for comparative purposes, an extended study of the susceptibility to acute alcoholization in the eggs of *Fundulus* prior and subsequent to fertilization was made. This indicates that the periods before and shortly after fertilization are especially critical in the history of the egg, a fact which, if it holds true generally, is of considerable significance and interest. The latter portion of the paper deals with the treatment of the spermatozoa of *Fundulus*, and the results of this on the subsequent development in the eggs fertilized by them.

There are many advantages in favor of the method of treating the germ cells directly with alcohol; for here the problem is not one involving the secondary effects of the altered soma upon the germ plasm. The fish *Fundulus* provided what seemed excellent material for such direct experimentation. The short-lived nature of the sperm of this species is, however, a fatal obstacle to anything like an extended treatment of the male germ cell. On the other hand, if the eggs of this form are allowed to stand for a much greater period than one half hour after being stripped, the percentage of fertilizations is materially lowered and in some instances the cleavages rendered abnormal. Thus these two conditions necessarily make the period of treatment a short one. Since practically no work of this kind has been done on short length treatments with different chemical poisons, this feature alone of these experiments would seem to make them of value.

Much interest has recently centered in the treatment of the germ cells of a number of different types of animals and the resulting effect on the processes of development. Notable in this regard are the experiments of Stockard ('12, '13, '16) showing the effects of alcohol on the germ cells of mammals, and those of the Hertwigs ('11, '12, '13) on the effects of radium on some less

highly developed types. Reviews of the literature along this line are readily accessible; so only the immediately applicable part need be considered here.

Dungay ('13) has made a study of the effects of heat, alcohol, alkali and hydrochloric acid upon the spermatozoa of *Nereis* and *Arbacia*. He finds that all of these agencies have an injurious effect upon subsequent development in the eggs fertilized by the treated spermatozoa.

Oppermann ('13) has found that radium modifies the sperm of the trout. His results very closely parallel those of Oscar and Gunther Hertwig on the frog in that treatments of a more limited nature serve to very profoundly modify the normal processes of development, and to produce many abnormalities. More extended treatment acted to so completely alter the spermatozoön that it retained only its fertilizing power, the eggs developing by a type of parthenogenesis.

In the same year Gunther and Paula Hertwig ('13) report that by treating the spermatozoa of Gobius jozo with 0.02 per cent. and 0.1 per cent. methylen blue for an hour, marked abnormalities occur in the development of normal eggs fertilized by spermatozoa thus modified. However, treatment of the spermatozoa of the same species for forty-five minutes with 0.1 per cent. solution of methylen blue showed no effect on these spermatozoa when used to fertilize the eggs of another fish, *Crenilabrus pavo*. This last fact makes the results obtained seem somewhat contradictory in nature.

The experiments reported in this paper were performed at the Marine Biological Laboratory, Woods Hole, Mass., during the summers of 1915 and 1916, and the writer wishes to express his appreciation to the director, Professor F. R. Lillie, and the authorities there for the facilities afforded him for the work. It is a pleasure, too, to acknowledge his indebtedness to Professor Charles R. Stockard for the suggestion of the problem and much helpful interest in the progress of the work.

Material and Methods.

The grades of alcohol used were dilutions of absolute alcohol of the best quality obtainable at the time. About half of the

material used was Kahlbaum's; the other was Eimer and Amend's absolute alcohol. The experiments show no differences between the effects of the two grades. The sodium hydroxide solutions used for activating the spermatozoa and for treating the eggs were dilutions of carefully titrated normal solutions.

In treating the eggs prior to fertilization, they were stripped and placed immediately in Syracuse watch glasses filled with the strengths of alcohol to be used. At the end of the period of treatment, they were washed out into finger bowls to which a quantity of sea water was twice added and decanted. Then the eggs were removed to a clean watch glass and fertilized with fresh spermatozoa, the mixture being allowed to stand for about fifteen minutes. They were then returned to a clean finger bowl of sea water. The washing lasted for a period of about five minutes, since in each series a number of lots of eggs was involved. With each experiment a control was operated, this being fertilized at the same time as the treated eggs.

In the experiments on the sperm, one tenth of a cubic centimeter of the grade of alcohol used was measured from a pipette into a Syracuse watch glass. A male *Fundulus* which had been carefully dried in a towel to remove all water from the surface of the body, was stripped and the drop of milt pressed into the fluid in the dish. The suspension was then stirred so as to render the distribution of the spermatozoa uniform. At the end of the period of treatment, the eggs to be fertilized were poured over the treated mass of spermatozoa. It was found that a high percentage of fertilizations always results in the control by leaving the eggs for five minutes in the drop of milt; so, in these experiments on the treatment of the spermatozoa, the whole series, with few exceptions, were allowed five minutes' contact with the mass of sperm cell for fertilization. In each experiment the influence of the alcohol on the egg alone was controlled by a lot of eggs treated with the same strength prior to fertilization.

Treatment of the Female Germ Cells.

1. *Prior to Fertilization.*

(*a*) *Condition of the Egg at this Period.*—At the time of laying, the egg of *Fundulus* is in many ways in a critical period of its

history. The important and delicate processes of maturation are in progress preparatory to fertilization. The character of its protective membranes has not yet been strengthened by the formation of the fertilization membrane. So, it might well be expected that profound effects may be produced at this time by what under other conditions would be considered slight cause. This seems to be quite clearly the case in the treatment of the eggs of *Fundulus* prior to fertilization.

(*b*) *Effects of Delayed Fertilization on Control Eggs.*—Upon examining the data given in the accompanying table (see Table I.) one might easily be led from the record of the control into the error that the eggs dealt with were a poor lot. The only indication of this is in the very much lowered percentage of fertilizations; for the number of sub-normal individuals, except in few instances, is slight. It seems clear, therefore, that if the eggs of *Fundulus* are allowed to stand for some twenty to thirty minutes before fertilization, because of the closure of the micropyle, or for some other reason, a number do not fertilize. These same eggs fertilized as soon as stripped give some seventy-five to ninety per cent. of fertilizations. The fact that the controls were fertilized at the same time as the treated lots serves, however, to place this factor upon the same basis in the comparison of results.

(*c*) *Effects of Treatment on the Viability of Eggs.*—In the experiments presented in Table I. and summarized in Table II., the first four series were treated for fifteen minutes prior to fertilization; the remainder were treated for twenty minutes. It will be noted that no developing individuals were secured from the eggs treated with strengths of alcohol higher than ten per cent., and only one in treatments of this percentage. These higher concentrations produced in many instances a shrinkage and distortion of shape in the egg, and of course the highest grades killed or fixed a number of the eggs.

In the case of those treated with two per cent. and five per cent. alcohol, the number of developing individuals is lowered several hundred per cent. as compared with the controls. This result is uniform throughout the whole series. In two instanecs, no individuals developed in the treatments with five per cent. alcohol.

(*d*) *Effects of Treatment on Cleavage.*—The "percentage developing beyond early cleavages" as applied in these results is a term which does not indicate the percentage of fertilizations except in the control. Here there are very few of the aberrant cleavages, though occasionally one does occur. However, in practically every series of eggs treated with two per cent. or five per cent. alcohol there occur some four or five hours after fertailiztion a number of aberrant cleavages. In some instances there may be as many as twenty or thirty of these in a single lot of eggs; in others only a very few.

The number of eggs developing in each lot treated is so small that it was rather difficult to follow the rate of cleavage as compared with the control. In the treated lot there were usually to be observed at the time of the first cleavage in the control a few in a similar stage. As will be noted from the legend accompanying the figures (see Figs. 1–9), the aberrant cleavages of the types figured in the text represent delayed cleavages in almost every instance.

In three series of experiments, all of these aberrant cleavages were removed to a separate dish. Upon examination several hours later the blastodiscs of the most of them were found to have disintegrated and to resemble the condition of the eggs that had not been fertilized at all. Frequently two or three out of some eighteen or twenty continued to develop at a slow rate, producing in most instances defective individuals or those of low vigor.

Sections were cut of several of these irregular cleavages, but aside from the fact that fragmented nuclear material of some sort seems to be present, the time available has prevented a fuller analysis. The cleavage planes are not deeply cut into the cytoplasm of the blastodisc, but are superficial in extent. It may be that the effect of the alcohol upon the egg nucleus is such that it causes fragmentation, followed by various degrees of rounding up of the adjacent cytoplasm. Other possibilities suggest themselves. The nature of these cleavages seems to afford an interesting problem, and one of which the writer hopes to make a closer study at some later date.

(*e*) *Effects on Percentage Developing Sub-normally.*—In these

TABLE I.

SHOWING EFFECTS OF TREATING EGGS WITH SEA WATER SOLUTIONS OF ALCOHOL FOR SHORT PERIODS PRIOR TO FERTILIZATION.

Percentage of Alcohol.	Total Number of Eggs in Lot.	Number Developing Beyond Early Cleavages.	Percentage Developing.	Number Developing at End of Six Days.	Total Number Sub-normal.	Percentage Sub-normal.
Series I.						
Control....	294	67	22.8	67	0	0
2.....	135	10	7.4	9	9	90
5.....	103	4	3.9	4	2	50
10.....	112	0	0	0	0	—
15.....	101	0	0	0	0	—
20.....	91	0	0	0	0	—
25.....	110	0	0	0	0	—
30.....	135	0	0	0	0	—
50.....	140	0	0	0	0	—
Series II.						
Control....	142	32	22.5	32	0	0
2.....	129	6	4.7	6	1	16.7
5.....	186	5	2.7	1	4	80
10.....	101	1	0.99	0	1	100
15.....	97	0	0	0	0	—
Series III.						
Control....	142	82	57.7	77	5	6.1
2.....	129	11	8.5	10	3	27.2
5.....	151	2	1.3	1	2	100
Series IV.						
Control....	126	36	28.6	33	3	8.3
2.....	108	2	1.8	0	2	100
5.....	123	0	0	0	0	—
Series V.						
Control....	163	75	46	71	4	5.3
2.....	224	8	3.5	8	3	37.5
5.....	171	0	0	0	0	—
10.....	176	0	0	0	0	—
Series VI.						
Control....	212	54	25.4	54	0	0
2.....	248	4	1.6	4	2	50
5.....	250	2	0.8	2	1	50
10.....	116	0	0	0	0	—
Series VII.						
Control....	99	40	40	38	2	5
2.....	134	5	3.7	5	3	60
5.....	123	2	1.6	1	2	100

experiments the developing eggs were separated from the undeveloping just as soon as practicable. In the treated eggs this meant during the first day, and in the control, not later than the second day. Thus it was possible to keep up with all of the eggs in the lot with accuracy. Observations were recorded in most instances twice a day in the earlier part of the experiment; and at least once a day thereafter until its conclusion. This means that those eggs which were weakened in vitality due to natural

causes and as the result of treatment are included in the percentage sub-normal recorded. Since development in this fish is well advanced at the end of the sixth day—circulation is well established by the third or fourth day, and the eyes well formed by the end of the sixth day—this is the period at which most of the experiments were terminated. This was done for practical experimental purposes, since to record observations on so large and rapidly accumulating a series would be almost an impossibility.

TABLE II.

SHOWING COMBINED RESULTS OF THE SEVERAL EXPERIMENTS ON EGGS PRIOR TO FERTILIZATION.

Percentage of Alcohol.	Total Number of Eggs in Lot.	Number Developing Beyond Early Cleavages.	Percentage Developing.	Number Developing at End of Six Days.	Total Number Sub-normal.	Percentage Sub-normal.
Control.....	1176	386	32.8	372	14	3.7
2.....	1107	46	4.1	42	23	50
5.....	1107	15	1.3	9	11	73.3
10.....	505	1	0.2	0	1	100

Examination of the accompanying table (see Table II.) will show that the percentage of sub-normals in the controls scarcely averages four per cent. In the dishes that were treated with two per cent. alcohol this varied from sixteen per cent. sub-normal to as high as ninety, with an average of fifty per cent. In almost every lot there were at least a few which came through their development normally. It was also frequently noted that where in earlier development every individual in a dish appeared abnormal, later on with circulation successfully established, the early deformity became regulated, and at the end of the sixth day the individual was included among the normal.

The effects of the five per cent. alcohol were more deep seated. In some instances only one individual came through to the end of the sixth day, and this was very abnormal. The types of defects produced in these treatments are considered in another part of this paper.

2. *Immediately after Fertilization.*

(*a*) *Condition of Egg at this Period.*—It has been shown by Loeb ('13) and others that the entrance of the spermatozoön in

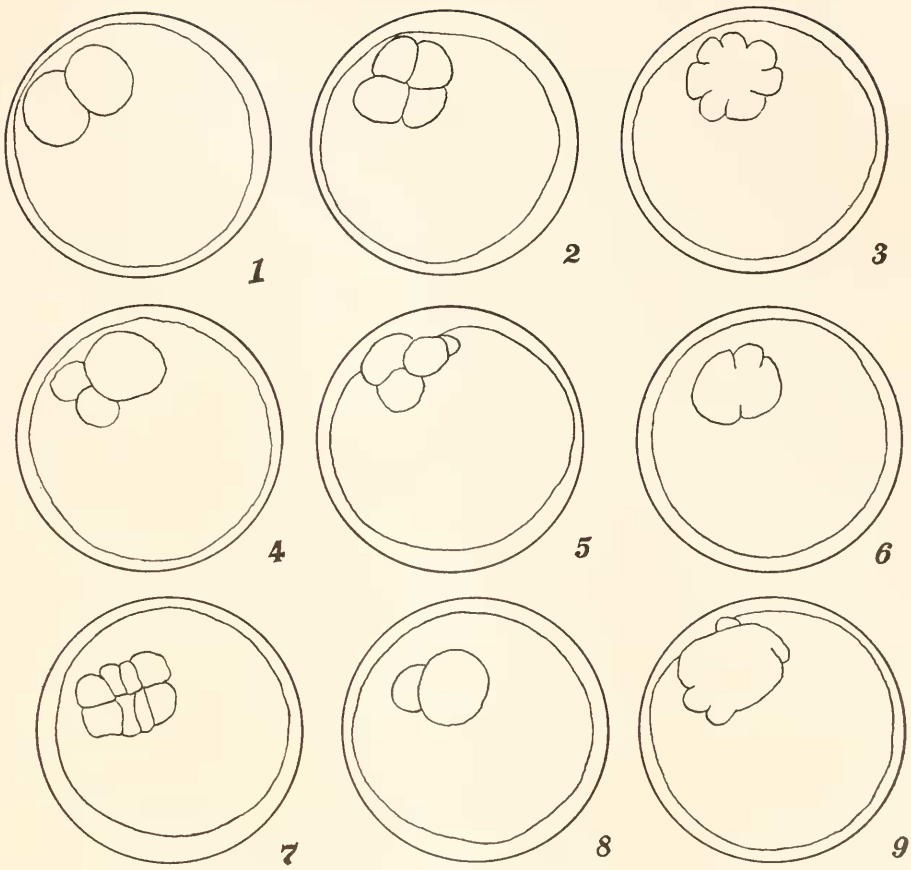


FIG. 1. A normal two-cell stage of *Fundulus* egg drawn to same scale as other cleavages.

FIG. 2. A normal four-cell stage.

FIG. 3. An aberrant cleavage occurring five hours after fertilization from egg treated with five per cent. alcohol in sea water prior to fertilization with norma sperm.

FIG. 4. An abnormal cleavage of an egg which was fertilized by sperm treated with ten per cent. alcohol in sea water.

FIG. 5. An irregular cleavage occurring three hours after fertilization in an egg which was treated with three per cent. alcohol in sea water for fifteen minutes prior to fertilization with normal sperm.

FIG. 6. An imperfect cleavage occurring four hours and a half after fertilization in an egg which was treated with two per cent. alcohol in sea water for fifteen minutes prior to fertilization with normal sperm.

FIG. 7. A normal eight-cell cleavage stage.

FIG. 8. An abnormal cleavage occurring two and a half hours after fertilization in an egg treated with two per cent. alcohol in sea water prior to fertilization with normal sperm.

FIG. 9. An aberrent cleavage occurring four hours after fertilization in an egg treated with five per cent. alcohol for twenty minutes prior to fertilization with normal sperm.

the process of fertilization instantaneously effects a marked change in the membrane of the egg. The permeability of this to certain substances seems to be increased, thus adapting the young developing individual to the interchange of materials which is necessary to its development.

There is also at this time a critical period in the nuclear phenomena. The male pronucleus has entered and is becoming acclimated to the new environment of the egg cytoplasm. The maturation divisions are being concluded and the female pronucleus preparing for union with the male pronucleus and the formation of the first cleavage spindle. These things indicate a great increase in the metabolic activity of the egg.

(*b*) *Effects of Higher Percentages on Development.*—It seemed that if the effects of treatment prior to fertilization were so decided, it would be well to test the period immediately after fertilization. Accordingly, a lot of eggs were subjected twenty minutes after fertilization to a graded series of alcohol to as high as twenty-five per cent. The results of this experiment are given in an accompanying table (see Table III.). Here it will be

TABLE III.

RESULTS OF TREATMENT OF EGGS WITH SEA-WATER SOLUTIONS OF ALCOHOL FOR FIFTEEN MINUTES AT TWENTY MINUTES AFTER FERTILIZATION.

Percentage of Alcohol.	Total Number of Eggs in Lot.	Number Developing Beyond Early Cleavages.	Percentage Developing.	Number Developing at End of Six Days.	Total Number Subnormal.	Percentage Subnormal.
Control	117	86	73.5	86	1	1.1
1	121	96	79.3	95	2	2
2	136	108	79.4	107	8	7.4
5	116	86	74.1	86	7	8.1
10	131	103	78.6	103	41	40
15	119	96	80.6	96	77	80
25	116	30	25.9	24	23	77

noted that the lower percentages produce an effect that is at least noticeable as compared with the control. However, it is necessary to use percentages of ten per cent. and over to secure the same effects at this period as are produced by two per cent. prior to fertilization.

The process of development is retarded by these treatments, and particularly so in the higher percentages. In the first

cleavage stage, only a small percentage were observed to be cleaving in these when the control showed a large proportion. It will be noted though that in all of the dishes except the one treated with twenty-five per cent. alcohol the percentage developing is as good as that of the control. A number of eggs were prevented from developing at all by treatments of this higher strength.

The types of defects secured by these short length treatments are much the same as those reported by Stockard ('10) from longer treatments of the eggs with lower percentages and beginning in the early cleavages. This is particularly true with the ten and fifteen per cent. strengths used.

3. *One Hour after Fertilization.*

The eggs of *Fundulus* cleave normally about two hours after fertilization. At one hour after fertilization, preparations must be well along in the cell for the formation of the first cleavage figures. The eggs seem considerably more resistant at this period as reference to the accompanying data will indicate (see Table IV.). This is no doubt due in part to the altered character

TABLE IV.

RESULTS OF TREATMENT OF EGGS WITH SEA-WATER SOLUTIONS OF ALCOHOL FOR ONE-HALF AND ONE HOUR AT ONE HOUR AFTER FERTILIZATION.

Series I. (treated for one-half hour).

Percentage of Alcohol.	Total Number of Eggs in Lot.	Number Developing Beyond Early Cleavages.	Percentage Developing	Number Developing at End of Six Days.	Total Number Subnormal.	Percentage Subnormal.
Control	93	74	79.6	70	6	8.1
2	91	69	75.8	69	7	11.5
5	69	51	73.9	50	1	1.9
10	72	61	84.7	61	0	0
15	69	51	73.9	51	5	9.8
25	103	60	58.2	60	6	10 [1]

Series II. (treated for one hour).

Control (same as Series I.).						
2	74	59	79.6	57	6	10
5	80	60	75	58	10	16.7
10	73	57	78	56	2	3.5
15	100	60	60	58	8	13.3
25	85	6	7	6	4	66.7

[1] Most of the individuals of this lot were considerably weakened as compared with the control but not sufficiently to be classed as defective.

of the egg membranes. Also the nuclear material must be more deeply imbedded in the cytoplasm than in an earlier period, and thus less in position to be affected.

The treatment of the lot of eggs used at this time show that even the ten and fifteen per cent. solutions acting for a half hour do not produce a much greater effect than do the two per cent. and five per cent. solutions at an earlier period after fertilization. In the treatments for one hour, the fifteen per cent. and twenty-five per cent. solutions show a considerable effect in reducing the percentage developing; particularly is this true in the case of the latter concentration, which killed all of the eggs except six. Even with this rigorous treatment two individuals of the six went through to the sixth day as normal individuals and from all appearances would have hatched in about the normal time.

While the data at hand are not as extensive as perhaps might be desired, it seems safe to draw the conclusion that the eggs of *Fundulus* are very susceptible to toxic effects shortly after fertilization and become more resistant as development proceeds.

4. *Effects of Dilute Sodium Hydroxide Solutions.*

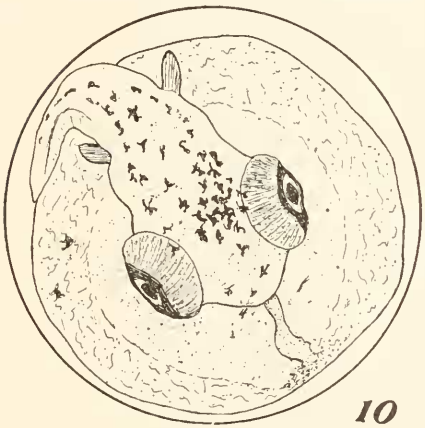
In several instances eggs were treated with dilutions of a standard alkali solution. The results show in some instances a large percentage of defective individuals from concentrations as low as $N/400$, $N/500$, and $N/800$ NaOH. The percentage of fertilizations is considerably lowered as the result of such treatments, and many aberrant cleavages occur. The effects in this connection, however, seem very variable and the time was not available for more than a few experiments, the data from which permit of no decided conclusions further than that some eggs are markedly affected in their development by treatments of low concentrations of alkali solutions. During last summer a lot of eggs treated with several drops of a very dilute solution of sodium hydroxide (0.6 c.c. $N/10$ NaOH + 50 c.c. sea water) for only fifteen minutes at twenty-five minutes after fertilization produced a large percentage of striking defects. One of the individuals of this lot is figured in the text (Fig. 14). The evidence is sufficient to state that the eggs of *Fundulus* in some instances in the more critical periods in their history before and after

fertilization may be much upset in their development by these alkali solutions of relatively low concentrations.

5. *Types of Defects.*

It is interesting and significant to note that treatments of the eggs for only fifteen and twenty minutes prior to fertilization produce most of the defective types secured by treatments for long periods, twenty-four hours or more, beginning in the early cleavage stages. Stockard ('10) has so thoroughly described these alcoholic types that it is only necessary here to refer to his paper on the effect of alcohol on the development of *Fundulus*.

Some of the type monsters secured are figured in the text. Several anophthalmic monsters were produced, one of those



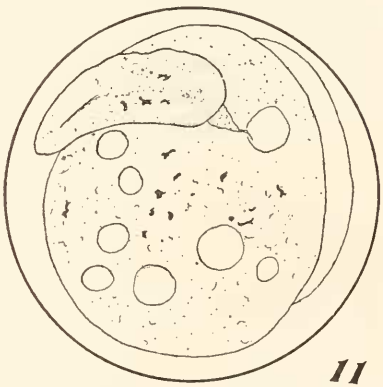


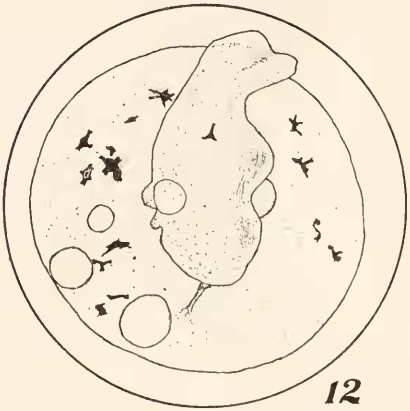
FIG. 10. A normal embryo six days old drawn to same scale as monsters.

FIG. 11. An anophthalmic monster of four days from egg treated with two per cent. alcohol in sea water prior to fertilization with untreated sperm.

figured showing a condition of spina bifida at the posterior end (Fig. 12). One or two cases of asymmetricum monophthalmicum (Fig. 13) occurred. Some showed the eyes curiously turned under towards each other on the ventral surface. Very few cases of cyclopia occurred, and these in eggs that showed an unusually large number of defective individuals, and it would be inferred that were in a very critical period at the time of treatment.

Quite a large proportion were generally defective, with much

shortened bodies, small eyes, a poor development of pigment, and a feeble circulation. A great assortment of defective individuals was produced in the lot of eggs treated with fifteen per cent. alcohol twenty minutes after fertilization, representing almost all of the types discussed by Stockard ('10). One of the microphthalmic monsters so developed is figured in the text (Fig. 17).



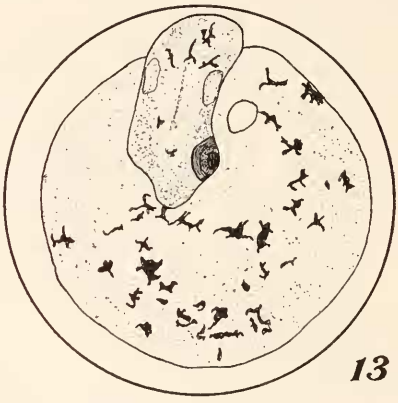


FIG. 12. An embryo six days old treated with two per cent. alcohol in sea water for fifteen minutes prior to fertilization with untreated sperm. A spina bifida, anophthalmic, with scarcely any pigmentation, and with a defective circulation.

FIG. 13. An asymmetricum monophthalmicum monster six days old from egg treated with two per cent. alcohol for fifteen minutes prior to fertilization with untreated sperm.

The term defective has come to signify to the mind a monster of an extreme type. So, in the tables and in many of the references in this paper the word sub-normal has been substituted. This is necessary in order to convey accurately the idea desired; for many of the individuals of the treated lot are those of much lowered vigor without any further special defect. Many embryos would not reach the six-day stage, but would disintegrate, the eggs becoming cloudy before that time. In a few instances after reaching about a late blastula or gastrula stage the embryo would slowly disintegrate. Thus it is to be noted that all grades of defective individuals occurred from aberrant cleavages to marked monsters of specific types and others which were merely slow in their rate of development and small in size.

IV. Treatment of Male Germ Cells.

1. *Length of Life of the Sperm of Fundulus.*

As has already been stated the length of life of the sperm of *Fundulus* after being stripped into sea water is surprisingly short. Newman ('06) has recorded that there is a definite spawning act in the breeding behavior; and in the light of this fact, the adaptiveness of this brief span of life is more readily understood. In this spawning act the copulating pair are in such position

Fig. 14. An embryo eleven days old which was treated twenty-five minutes after fertilization for fifteen minutes with dilute alkali solution (0.6 c.c. $N/10$ NaOH plus 50 c.c. sea water). A cyclopean monster, embryo twisted and short, with heart beat, and no circulation.

Fig. 15. A sixteen-day embryo developing from egg which was fertilized by sperm treated with fifteen per cent. alcohol in distilled water for three minutes, and afterwards activated with a weak alkali solution (0.6 c.c. $N/10$ NaOH plus 50 c.c. sea water). Microphthalmic monster, deformed in shape, yolk sac imperfectly absorbed, heart beat with no circulation.

that immediately upon the extrusion of the eggs the synchronous rhythmic discharge of the milt from the male occurs, and this places the spermatozoa in direct contact with the eggs to be fertilized.

It was necessary at the outset of a series of experiments of the character herein discussed to study the length of life of the sperm in various solutions. From the accompanying table (see Table V.) it will be noted that the duration of activity in a small drop —one tenth of a cubic centimeter—of distilled water is about one

minute or less. This factor held constant in the alcohol solutions in distilled water to as high as twenty per cent. In the earlier part of the experiments of the season of 1915, the spermatozoa were treated with the following strengths of alcohol in distilled water: 0.1 per cent., 0.2 per cent., 0.5 per cent., 1 per cent., 1.5 per cent., 2 per cent., 2.5 per cent., 2.8 per cent., 3 per cent., 3.5 per cent. and 4 per cent. At the end of a minute, in most instances, the spermatozoa were inert and consequently incapable of fertilizing an egg. It was found possible to activate them,

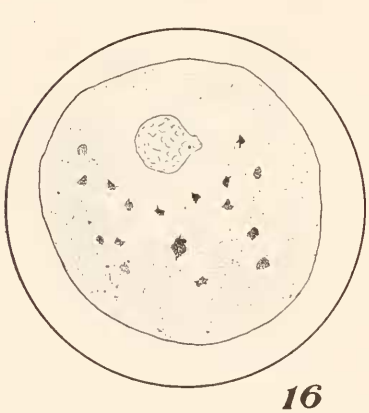


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FIG. 16. An embryo from same lot as the one in preceding figure with only remains of embryo disintegrating over yolk mass.

FIG. 17. A microphthalmic monster of six days treated with fifteen per cent. alcohol in sea water for fifteen minutes at twenty minutes after fertilization.

however, by the use of a weak sodium hydroxide solution (0.6 c.c. $N/10$ NaOH + 50 c.c. sea water, or better, a somewhat stronger solution 6 drops $N/10$ NaOH + 10 c.c. sea water). Just a drop of such a solution set the spermatozoa active in a short time, and in the treatments with the lower concentrations of alcohol, they were enabled to fertilize the eggs with a considerable percentage developing.

A large proportion of the spermatozoa could be activated in the treatments of lower concentrations and continue active for several minutes. It became evident therefore that best results were likely to be obtained by treatments of fifteen and twenty per cent. solutions. It was found possible to treat the spermatozoa

with twenty per cent. alcohol for even three minutes and activate a sufficient number to get some eggs fertilized. The small proportion which were revived to activity in this way indicated that most of the spermatozoa had been injured to a sufficient degree to cause their death.

Sea water solutions of alcohol produced very different results. The length of life was much longer in these, but except in rare instances, not a single spermatozoön after having ceased movement could be activated with the addition of the alkali solution. The resistance of the spermatozoa from a number of fish to the action of several solutions is given in the accompanying table (see Table V.). It will be observed that only in the highest percen-

TABLE V.

LENGTH OF LIFE OF SPERM OF *Fundulus* IN ONE TENTH OF A CUBIC CENTIMETER OF DISTILLED WATER, SEA WATER, AND GRADES OF ALCOHOL IN SEA WATER.[1]

Distilled Water.	Sea Water.	1 Per Cent. Alcohol.	2 Per Cent. Alcohol.	5 Per Cent. Alcohol.
1 min.	10 mins.	12 mins.	9 mins. 30 secs.	9 mins. 30 secs.
1 min. 10 secs.	8 mins.	8 mins.	8 mins.	9 mins.
45 secs.	14 mins.	10 mins.	8 mins. 30 secs.	9 mins.

10 Per Cent. Alcohol.	15 Per Cent. Alcohol.	20 Per Cent. Alcohol.	25 Per Cent. Alcohol.
8 mins.	5 mins. 30 secs.	2 mins.	killed at once.
9 mins.	8 mins. 30 secs.	3 mins.	30 secs.
7 mins. 30 secs.	7 mins.	2 mins. 30 secs.	killed at once.

tages is the time element satisfactorily eliminated. In these the greatest length of life is less than the shortest length found in the sea water alone. On this account, it was with these higher percentages that much of the work on the male germ cells was done.

The factor of dilution is another important one in the length of life of the sperm. A mass of spermatozoa placed in a large quantity of pure sea water became inactive in a very short time. In order to control the effect of dilution, the amount of solution used in these experiments was carefully measured and constant.

The causes of the inactivity of the sperm in distilled water,

[1] These periods represent the interval between the stripping of the milt from the male and the cessation of movement on the part of every spermatozoön in the dish. Consequently they represent the length of life of the most vigorous sperm in each lot.

and the striking behavior in activation with a dilute alkali afford a rather interesting problem. It is one of considerable complexity, related as it is so intimately with the mechanics of motility in the spermatozoön. The osmotic differences due to the behavior of different types of electrolytes is involved, and this is rather aside from the questions aimed at in this paper.

The main difficulty confronting one in the injury of the spermatozoa is to get such a dose and period of exposure as to cripple the spermatozöon and at the same time not deprive it of its motility and power of fertilization. Radium seems to do this to an excellent degree in some forms, but the matter is a much more difficult one with solutions of alcohol.

2. *Experiments on Treatment of Spermatozoa.*

In considering the experiments with the spermatozoön the problem of the differentiation of the effects of the alcohol on the spermatozoön and on the egg must be kept in mind. It was impossible to separate the treated spermatozoa from the treating agent and have them retain their fertilizing capacity. Yet as has been shown in the first part of this paper, low concentrations of alcohol, acting for short periods before and after fertilization, materially affect the developing eggs. Several factors served to reduce this effect, and on a comparative basis it would seem to eliminate it by establishing a differential.

It was found that five minutes' exposure of a control to the action of the sperm served to insure about as good a fertilization result as when allowed to stand for fifteen minutes. So with the exception of the experiment with the twenty per cent. solution of alcohol in distilled water, all of the eggs fertilized by treated spermatozoa, as well as the controls, were given five minutes for fertilization. The object of this was to reduce to a minimum the length of time that the alcohol acted on the eggs.

Then, again, over a hundred eggs carrying their surrounding fluid and some sea water from the bodies of the fish during the act of stripping served to bring to a very dilute percentage the one tenth of a cubic centimeter of alcohol used. In the case of the treated mass of sperm cells, the alcohol was diluted not only by the milt of the male, but also by the fluid of the eggs added.

Two controls were operated in each case. One of these consisted in normal eggs fertilized with untreated spermatozoa. In the other the eggs were treated with an equal amount of alcohol to that used in the other dishes of the experiment. They were then washed and fertilized with untreated spermatozoa. Such treatment as this should, for reasons mentioned above, quite fully meet the requirements of a control.

An attempt was made to alcoholize the males. Several of these were placed in various concentrations of alcohol, but the percentage in which they will live for any length of time is so dilute that this method was soon abandoned. The difficulties of acclimatization and of proper aëration seem to render this sort of experiment an impractical one.

(*a*) *Effects of Twenty Per Cent. Alcohol in Distilled Water.*— The spermatozoa treated with two tenths of a cubic centimeter of this strength of alcohol became motionless in less than a minute. They were allowed to remain in the solution for two and three minutes. Then they were activated with a dilute solution of sodium hydroxide. The lot of eggs were rather weak to start with, as is indicated by the twenty per cent. sub-normal which occurred in the control (see Table VI.). However, the number

TABLE VI.

EFFECTS OF TWENTY PER CENT. DISTILLED WATER SOLUTION OF ALCOHOL ON THE SPERM OF *Fundulus*.

Treatment	Total Number of Eggs in Lot	Number Developing Beyond Early Cleavages.	Percentage Developing.	Number of Aberrant Cleavages Subsequently Disintegrating.	Number Developing at End of Sixth Day.	Total Number Sub-normal.	Percentage Sub-normal.
Control	103	77	74.7	0	64	16	20.7
Eggs treated with 0.2 c.c. of 20 per cent. alc. + 0.2 c.c. alkali[1] for 15 minutes	106	13	12.2	3	6	7	53.7
Spermatozoa treated with 0.2 c.c. of 20 per cent. alcohol for 3 minutes and activated with 0.2 c.c. alkali	152	0	0	9	0	0	—
Same treatment of sperm except for two minutes	128	8	6.2	9	5	3	37.5
Same treatment of sperm for two minutes	116	7	6	5	3	7	100

[1] The strength alkali used for activating was six drops of $N/10$ NaOH + 10 c.c. sea water.

TABLE VII.

SHOWING EFFECTS OF FIFTEEN PER CENT. ALCOHOL IN SEA WATER SOLUTION UPON THE SPERM OF *Fundulus*.

Treatment.	Total Number of Eggs in Lot.	Number Developing Beyond Early Cleavages.	Percentage Developing.	Number Developing at End of Sixth Day.	Total Number Subnormal.	Percentage Subnormal.
Series I.						
Control	61	25	41	24	1	4
Eggs treated for five minutes prior to fertilization in 0.1 c.c. 15 per cent. alcohol	77	15	19.4	15	0	0
Sperm treated with 0.1 c.c. 15 per cent. alcohol for 5 minutes	145	77	53.1	72	5	6.5
Series II.						
Control	163	62	38	58	4	6.5
Eggs treated for five minutes prior to fertilization in 0.1 c.c. 15 per cent. alcohol	119	20	16.8	13	7	35
Sperm treated with 0.1 c.c. 15 per cent. alcohol for 1 minute	194	22	11.3	16	6	27.3
Sperm treated with 0.1 c.c. 15 per cent. alcohol for 5 minutes	149	5	3.4	4	2	40
Series III.						
Control	57	28	49.1	28	0	0
Eggs treated for five minutes prior to fertilization in 0.1 c.c. 15 per cent. alcohol	65	31	47.7	31	0	0
Sperm treated with 0.1 c.c. 15 per cent. alcohol for 1 minute	62	42	67.7	42	3	7.1
Sperm treated with 0.1 c.c. 15 per cent. alcohol for 6 minutes	94	26	27.6	26	1	3.8
Series IV.						
Control	62	27	43.5	25	3	11.1
Eggs treated for five minutes prior to fertilization in 0.1 c.c. 15 per cent. alcohol	61	9	14.7	9	1	11.1
Sperm treated with 0.1 c.c. 15 per cent. alcohol for 1 minute	102	25	24.5	21	6	24
Sperm treated with 0.1 c.c. 15 per cent. alcohol for 7 minutes	121	3	2.5	3	0	0

of eggs represented and the fact that they were all from the same lot seems to eliminate this objection.

Where the spermatozoa were treated for three minutes, none of the eggs fertilized developed beyond the irregular early cleavages. There were nine of these, all of which subsequently disintegrated. Of the eggs fertilized with those treated for two minutes, one lot had five developing at the end of six days, all of which were normal. However, three of the eight which passed the early cleavage stages had disintegrated by the fifth day; and there

were nine aberrant cleavages which early disintegrated. Another lot similarly treated showed even more decided effects; for of the seven developing beyond the early cleavages, all were defective at the end of the sixth day.

These results seem to warrant the conclusion that a treatment of this strength is injurious to the sperm as well as to the egg. This is most clearly indicated in the instance where the spermatozoa were treated for three minutes.

(*b*) *Effects of Fifteen Per Cent. Alcohol in Sea Water.*—The effects of this strength solution are not so marked either on the eggs or on the sperm as was the solution used in the experiments just discussed. In two instances there were no sub-normal individuals developing from the lot of eggs treated for five minutes prior to fertilization, though in most of the dishes of eggs so treated, the percentage developing was considerably below that of the control. The percentage sub-normal in Series I. (Table VII.) as the result of treating the sperm for five minutes is not very appreciably different from that in the control.

There is, however, a larger percentage developing in this dish; a fact which indicates a greater vigor on the part of the spermatozoa of this male, as well as perhaps a stimulating effect on the part of the alcohol. This is also shown by the accelerated cleavage on the part of the eggs in this dish, the early cleavages having occurred several minutes before those in the other dishes of the series.

In the second series (Table VII.) the treatment of the eggs prior to fertilization shows a decided effect, thirty-five per cent. developing sub-normally. The treatment of the sperm for one minute seems in this series to have less effect than the treatment of the eggs alone prior to fertilization. Out of the five developing beyond the early cleavages from the fertilizations with the spermatozoa treated for five minutes, two resulted in defective embryos.

In the other two series with this strength, the longer treatment seems to have a selective action on the sperm, a much smaller percentage of sub-normals occurring than in the one minute treatments. The strong spermatozoa seem to have been selected by resisting the treatment, and while the percentage developing

in these cases is much lowered, those that do develop seem to be more vigorous. This is indicated by the large percentage developing to the sixth day as normal individuals.

In the summarized results of these four experiments (see Table VIII.), it appears that the treatments of one minute

TABLE VIII.

SUMMARY OF RESULTS OF FIFTEEN PER CENT. ALCOHOL IN SEA WATER ON THE SPERM OF *Fundulus*.

Treatment.	Total Number of Eggs in Lot.	Number Developing Beyond Early Cleavages.	Percentage Developing.	Number Developing at End of Sixth Day.	Total Number Sub-normal.	Percentage Sub-normal.
Control	343	142	41.4	135	8	5.6
Eggs treated for five minutes prior to fertilization in 0.1 c.c. 15 per cent. alcohol	322	75	23.3	68	8	10.6
Sperm treated with 0.1 c.c. 15 per cent. alcohol for 1 minute	358	89	24.8	79	15	16.8
Sperm treated with 0.1 c.c. 15 per cent. alcohol for five or more minutes	509	111	21.8	105	8	7.2

duration have a greater effect than does the longer treatment. The shorter treatment seems to cripple the weaker spermatozoa, which fertilize the eggs and result in sub-normal individuals; while the longer treatment weeds out the weaker ones, the strongest ones being unaffected and leading to normal development when they fertilize the egg.

(*c*) *Comparative Effects of Treatments of Different Lengths.*—One method used in determining whether the treatment was effective on the spermatozoa was to give them equal doses for different lengths of time. In such an experiment the effect on the eggs should be a constant factor, and a differential thereby established between the injury of the spermatozoa treated for one minute and those treated for five minutes or longer. Five such series were carried out and the combined results are given in tabulated form (see Table IX.).

Examination of these data will indicate that while the percentage sub-normal is not very large in any instance, the greater effect is with the one minute treatment. The longer treatments

TABLE IX.

COMBINED RESULTS OF FIVE SERIES OF EXPERIMENTS SHOWING DIFFERENCES IN EFFECTS OF ONE MINUTE AND FIVE OR MORE MINUTES' TREATMENT OF THE SPERM OF *Fundulus* WITH FIFTEEN PER CENT. ALCOHOL.

Treatment.	Total Number of Eggs in Lot.	Number Developing Beyond Early Cleavages.	Percentage Developing.	Number Developing at End of Sixth Day.	Total Number Subnormal.	Percentage Subnormal.
Control	495	213	43.3	206	9	4.2
Sperm treated with 0.1 c.c. 15 per cent. alcohol for 1 minute	607	204	33.6	191	31	15.1
Sperm treated with 0.1 c.c. 15 per cent. alcohol for five or more minutes	648	43	6.6	42	3	7

lead to the elimination of the weaker lot, and while the percentage developing is much smaller, subnormal individuals are rarer than in the control.

(*d*) *Selective Action with Treatment of Ten Per Cent. Alcohol.*— Three separate lots of spermatozoa were treated with two tenths of a cubic centimeter of ten per cent. alcohol in sea water for such a length of time that only a few were left active in the dish. A lot of eggs were then mixed with the treated drop of milt and allowed to stand five minutes for fertilization. In one instance

TABLE X.

SHOWING THE EFFECTS OF TEN PER CENT. ALCOHOL IN SEA WATER ON THE SPERM OF *Fundulus*.

Treatment.	Total Number of Eggs in Lot.	Number Developing Beyond Early Cleavages.	Percentage Developing.	Number Developing at End of Sixth Day.	Total Number Subnormal.	Percentage Subnormal.
Control	127	81	63.7	81	1	1.2
Eggs treated for five minutes prior to fertilization with 0.2 c.c. of 10 per cent. alcohol	144	67	46.5	67	5	7.5
Sperm treated with 0.2 c.c. 10 per cent. alcohol for such a length of time as only a few were left active.	200	2	1	2	0	0
Sperm treated with 0.2 c.c. 10 per cent. alcohol for such a length of time as only a few were left active.	195	2	1	2	0	0
Sperm treated with 0.2 c.c. 10 per cent. alcohol for such a length of time as only a few were left active.	203	0	0	0	0	—

no fertilizations were secured; in the others, only two and these were developing normally at the end of the sixth day.

There seems to be very plainly exhibited in this experiment an eliminating action of the alcohol on the spermatozoa. The treatment was of sufficient length to kill out all of the weaker ones, and the stronger ones fertilized the eggs with resulting normal individuals.

(*e*) *Effects of Methylene Blue Solution on Eggs Prior to Fertilization.*—As stated earlier in this paper, the results of the Hertwigs ('13) on the treatment of the sperm with methylene blue seem somewhat contradictory in nature. Treatment of the spermatozoa of *Gobius jozo* for one hour in 0.02 per cent. and 0.1 per cent. solutions showed a decidedly injurious effect when used to fertilize the eggs of the same species of fish. When the spermatozoa were treated for forty-five minutes with the 0.1 per cent. solution and used to fertilize the eggs of another fish, *Crenilabrus pavo*, the resulting development showed no effect of this treatment.

In the light of the experiments reported in the first section of this paper, it appeared that the discrepancy might be explained as due to the difference in effect upon the eggs of the two fishes of the methylene blue in the sperm suspension used to fertilize the eggs of the fish.

TABLE XI.

RESULTS OF TREATING EGGS OF *Fundulus heteroclitus* WITH SOLUTIONS OF METHYLENE BLUE FOR FIFTEEN MINUTES PRIOR TO FERTILIZATION.

Percentage of Methylene Blue	Total Number of Eggs in Lot.	Number Developing Beyond Early Cleavages.	Percentage Developing.	Number Developing at End of Sixth Day.	Total Number Sub-normal.	Percentage Sub-normal.
Control	130	24	17	24	0	0
0.02 per cent. methylene blue in sea water	99	16	16.1	16	8	50
0.1 per cent. methylene blue in sea water	104	17	16.3	14	7	41.1
0.02 per cent. methylene blue in distilled water	131	46	35.1	46	5	10.8
0.1 per cent. methylene blue in distilled water	104	11	10.6	9	3	27.2

Accordingly an experiment was planned to test the plausibility of this view when applied to the eggs of *Fundulus heteroclitus*. The results of this experiment are given in an accompanying table (see Table XI.). While the matter was not exhaustively

followed out, the high percentage of sub-normals resulting seems to suggest that this is a factor that should at least be carefully controlled, and the effects they have reported as being due to the toxic action of the methylene blue on the sperm may very probably have been due in considerable part to the action of the methylene blue added with the sperm upon the eggs themselves.

While only a low percentage of the control eggs developed in this experiment—on account of the delayed fertilization—a very striking abundance of subnormal individuals were produced in the methylene blue series and no such specimens were recorded in the control. This fact would seem to indicate in a positive way the effects of such solutions on the development of the eggs. The eggs of *Fundulus* are very probably much more hardy and resistant to all treatments than are those of *Gobius*, judging from the experiments reported in the literature on the two forms.

Discussion.

The results reported in this paper seem to lead to rather definite conclusions. One of these is that the period in the egg prior to fertilization is a critical one. Dosages of alcohol which are comparatively negligible in their action an hour after fertilization produce marked effects at this earlier period. Also the period very shortly after fertilization seems another time at which the egg is more susceptible to injury.

In analyzing results such as these one is impressed with the number of factors involved. Different eggs at the same period in their development differ in their resistance to the same substances in solution. A lethal dose for one is comparatively slight in its effect upon another. A part of this results no doubt from the differences in permeability of the individual eggs in the lot involved due to their slightly different metabolic or developmental states. As a consequence more of the toxic substance passes through the membranes of certain eggs to affect their protoplasmic content than through the membranes of others.

Just before fertilization, the maturation processes are in operation in the egg. Immediately after fertilization these are being completed, and the male and female pronuclei are near the surface of the protoplasmic disc of the egg. The effect of

the treatments with alcohol at these periods would certainly be due in part to its effect on the nuclear material. The nature of this effect one can little more than surmise. Alcohol has an affinity for water, and would tend to remove this from the protoplasm of the egg. On the other hand, it may act to alter to some degree the actual chemical composition of the nuclear material.

One is led naturally to the position that in the case of the eggs of other forms, perhaps even of mammals, similar critical periods may exist. If so, this finding is an important one; for it may be that sudden acute intoxication of the female parent about the time of conception may lead to the abnormality of the resulting embryo, or may even prevent the fertilization of the egg. There are numerous instances cited to support such a view in medical statistics. A somewhat different statement of the case is that acute intoxication of the eggs at critical periods in their history may act to prevent development altogether, or to render it abnormal in a considerable proportion of cases.

The spermatozoa show a surprising degree of resistance to the action of alcohol solutions in both sea water and distilled water. Treatments with the higher percentages of alcohol yielded the best results. Here one could be reasonably sure that one was injuring the spermatozoa; for they lived a much shorter time in these concentrations than they do in pure sea water.

While the results are not as clean cut in the experiments on the sperm as in the case of the egg, there does seem to be in several instances a definite injury to the sperm. In others the action of the alcohol seems a selective one. Still the results here are much complicated by the action of the treating reagent on the egg prior and just subsequent to fertilization, even though the greatest care was taken to closely control this factor.

Summary.

1. The eggs of *Fundulus heteroclitus* are very susceptible to injury from treatment with low concentrations of alcohol prior to fertilization. This period seems an especially critical one in the history of the egg.

2. Two per cent. and five per cent. solutions of alcohol in sea

water acting for short lengths of time prior to fertilization reduce to a marked degree the number of eggs which develop.

3. Such treatment of the eggs of *Fundulus* produce a number of aberrant cleavages. These seem to be due in part to an effect upon the chromatin of the egg.

4. A large proportion of the individuals which develop from eggs treated with alcohol prior to fertilization are markedly defective.

5. Twenty minutes after fertilization the eggs are much more sensitive to injury with alcohol than at one hour after fertilization and in the early cleavages.

6. The types of defects produced by these acute treatments were of all grades from aberrant cleavages to marked monsters of specific types, and others which were merely slow in their rate of development and small in size.

7. Dilute solutions of sodium hydroxide acting prior to fertilization produced in some eggs much the same effects as secured from treatments with alcohol.

8. The spermatozoa of *Fundulus* usually continue active for less than a minute after being stripped in one tenth of a cubic centimeter of distilled water, and in solutions of alcohol in distilled water. After complete cessation of movement in these solutions, some of the spermatozoa may be activated with a dilute sodium hydroxide solution sufficiently to fertilize an egg.

9. In one tenth of a cubic centimeter of sea water and in the weaker concentrations of alcohol in sea water, the spermatozoa live for several minutes after being stripped. After cessation of movement in these solutions the spermatozoa very rarely activate upon the addition of an alkali solution.

10. The higher concentrations of alcohol acting for short periods on the spermatozoa seem to injure many of them without depriving them of their fertilizing power. When acting for longer periods, these same concentrations, in some instances, clearly eliminate the weaker spermatozoa and the resistant ones which survive are often capable of producing normal fertilization and development.

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